

RESEARCH ARTICLE

DNA damage induces expression of WWP1 to target Δ Np63 α to degradation

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Abstract

Δ Np63 α plays key roles in cell survival and proliferation. So its expression is always tightly controlled in cells. We previously reported that DNA damage down-regulates transcription of Δ Np63 α in FaDu and HaCat cells, which contributes to cell apoptosis. In the present study, we found that DNA damage induces down-regulation of Δ Np63 α via facilitating its proteasomal degradation in cell lines such as MDA-MB-231 and MCF10A. Further investigation revealed that transcription of WWP1 is stimulated by DNA damage in these cells. Knock-down of WWP1 abrogates DNA damage-induced down-regulation of Δ Np63 α and partially rescues cell apoptosis. Interestingly, DNA damage may stimulate WWP1 through different mechanisms in different cell types: it up-regulates transcription of WWP1 in a p53-dependent manner in MCF10A and HEK293 cells, while miR-452 may be involved in DNA damage-induced up-regulation of WWP1 in MDA-MB-231 cells. Our study demonstrates a novel pathway which regulates Δ Np63 α upon cellular response to chemotherapeutic agents.

Introduction

p63 gene is a homologue of p53 and p73 [1–3]. Owing to alternative transcription starting and splicing, p63 encodes a series of protein isoforms, including TAp63 α and Δ Np63 α . TAp63 isoform proteins contain a full and intact transactivation domain (TAD) each at their N-termini, which endows them with potent transactivities. Activation of Bax, p21, and other downstream target genes, mediated by TAp63s, results in cell cycle arrest or cell apoptosis. Δ Np63 isoforms possess a shorter and incomplete N-terminal TAD each, causing defect of transactivities in them. Δ Np63 α is the predominant p63 isoform, and has been reported to antagonize transactivities of p53, TAp63s and TAp73s via forming inhibitory heterogenous complexes with these proteins or competitive binding to promoters of their downstream targets [4–9]. This is consistent with evidence supporting that Δ Np63 α can enhance cell survival, growth and proliferation, though it has been recently reported that Δ Np63 α switches on transcription of genes such as MKP3 to modulate cancer metastasis and cell differentiation [3, 10–16].

In our previous study, we found that Δ Np63 α is a major player in DNA damage-induced cell apoptosis in two cell lines bearing mutant p53 gene, FaDu and HaCat [17]. Treatment with DNA damage drugs, doxorubicin (Doxo) and cisplatin (CDDP), induces down-regulation of Δ Np63 α and cell apoptosis. This down-regulation is independent of p53 and occurs mainly at transcription level. Knock-down of Δ Np63 α directly induces cell apoptosis and increases cellular sensitivity to DNA damage agents, while exotic expression of it promotes cell proliferation and confers cells resistance to DNA damage-induced apoptosis [17, 18].

In the present work, we found that DNA damage promotes protein degradation of Δ Np63 α in HEK293 cells transfected with Δ Np63 α , and two cell lines expressing high level of endogenous Δ Np63 α MDA-MB-231 and MCF10A. Further study revealed that transcription of WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) is induced by DNA damage. Knock-down of WWP1 abrogates Δ Np63 α destabilization and partially rescues cell apoptosis induced by DNA damage. Intriguingly, we found that DNA damage enhances WWP1 mRNA level in different manners in these cells: in MCF10A and HEK293 cells, DNA damage elevates p53 protein level to enhance transcription of WWP1, while in MDA-MB-231 cells, down-regulation of microRNA-452 may account for the increase in WWP1 after DNA damage treatment. Taken together, we report here a different mechanism of DNA damage-induced down-regulation of Δ Np63 α : except for decreased transcription of Δ Np63 α in some cells, which we have reported previously, DNA damage can also destabilize Δ Np63 α via stimulating its E3 ligase WWP1 in a p53-dependent or some other manners in some cell lines.

Materials and methods

Cell culture and transfection

HEK293(Δ Np63 α) [18] and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Hyclone). MCF-10A cells were grown in DMEM/F12 media (Hyclone), supplemented with 20 ng/ml epidermal growth factor (Invitrogen), 100 ng/ml cholera toxin (Sigma), 10 mg/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma), 1% penicillin/streptomycin (Hyclone) sulfate and 5% FBS (Hyclone). All cells were cultured at 37°C in a humidified 5% CO₂ incubator.

Small interfere RNA (siRNA) for p53 (1#sip53, Santa Cruz, sc-29435; 2#sip53, Santa Cruz, sc-44218) or WWP1 (1#siWWP1, GenePharma [18]; 2#siWWP1, Santa Cruz, sc-40366) and pre-miR microRNA precursor for microRNA-452 (Ambion, PM12509) [19] were transfected with Lipofectamine 2000 (Invitrogen) as described in the manufacture's instruction. 100 pmol of each siRNA or pre-miR was used per well of 6-well plate at 80~90% cell confluence.

Drug treatment and MTT assay

For DNA damage treatment, cell culture media were supplemented with 0.5 μ M Doxorubicin (Doxo, Sigma) or 20 μ M cisplatin (CDDP, Sigma) for 48 hours, and then subject to immunoblot, MTT assay, or qRT-PCR analysis. To inhibit proteasomal degradation of cellular proteins or measure protein half life, cells were treated with 10 μ M MG132 (Sigma) or 50 μ g cycloheximide (CHX, Sigma), respectively.

MTT assay was performed as previously described [17].

Immunoblot analysis (IB) and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Specific antibodies to p63 (4A4 mouse monoclonal antibody, Santa Cruz, Dallas, TX, USA, 1:200), actin (rabbit polyclonal antibody, Santa Cruz, 1:1000), WWP1 (rabbit monoclonal

antibody, Epitomics, Burlingame, CA, USA, 1:3000), p53 (DO-1 mouse monoclonal antibody, Santa Cruz, 1:250), and N-terminal cleaved PARP1 (rabbit polyclonal antibody, Zenable, Chengdu, China, 1:8000) were used for immunoblot analysis as described previously [18].

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for p63 and WWP1 was performed as described previously [17]. The expression level of miR-452 was analyzed with TaqMan qRT-PCR kit (assay ID: 002329) and normalized to RNU48 (assay ID: 001006) according to the manufacturer's instruction and as described previously [19].

Results

1. DNA damage promotes proteasomal degradation of ΔNp63α

We previously reported that ΔNp63α was significantly down-regulated in FaDu and HaCat cells after treatment with DNA damage agents, doxorubicin (Doxo) and cisplatin (CDDP); this regulation occurs mainly at mRNA level, while the half-life of ΔNp63α protein is slightly decreased [17]. To further investigate the effect of DNA damage on protein stability of ΔNp63α, we treated a HEK293 cell line stably expressing exogenous ΔNp63α [HEK293 (ΔNp63α)], which we generated previously [18], with proteasome inhibitor, MG132, in combination with either Doxo or CDDP. The results showed that either Doxo or CDDP significantly down-regulates ΔNp63α protein level which can be dramatically restored by MG132 (Fig 1A).

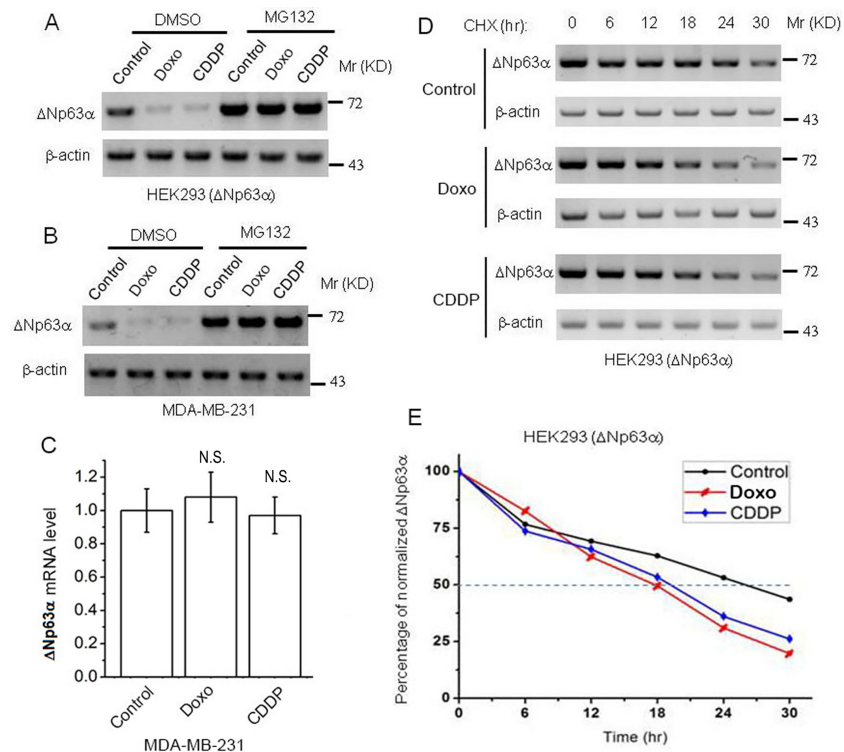


Fig 1. DNA damage promotes proteasomal degradation of ΔNp63α. Cells were treated with 1 μM Doxorubicin (Doxo) or 20 μM cisplatin (CDDP), plus 10 μM MG132 or its vehicle control DMSO, for 48 hours, then subject to immunoblot (IB) analysis (A and B) or qRT-PCR analysis (C). For measurement of protein half-life, cells were treated with 1 μM Doxo or 20 μM CDDP, plus 50 μg/mL cycloheximide (CHX) for indicated durations, then subject to IB analysis (D). Intensities of ΔNp63α bands in D were quantified with Image Lab (Bio-Rad) and normalized with β-actin bands (E). Expression level of ΔNp63α was normalized to β-actin and presented as means ± standard deviations (n = 3). Analysis with Student's *t* test reveals no significant difference (N.S.) between Doxo/CDDP-treated and control groups.

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Similar observation was found in MDA-MB-231, which is a human breast adenocarcinoma cell line expressing endogenous Δ Np63 α (Fig 1B). On the other hand, our results of quantitative reverse-transcription PCR (qRT-PCR) revealed that Doxo or CDDP does not significantly affect mRNA level of Δ Np63 α in MDA-MB-231 cells (Fig 1C). Our cycloheximide (CHX) chase results revealed that protein half life of Δ Np63 α is shortened by either Doxo or CDDP (Fig 1D and 1E). These results suggest that DNA damage promotes proteasomal degradation of Δ Np63 α .

2. mRNA level of WWP1 is stimulated by DNA damage

As an E3 ligase targeting Δ Np63 α to proteasomal degradation, WWP1 was previously reported to be induced by Doxo and CDDP [20]. In line with the previous reports, we observed that either Doxo or CDDP induced up-regulation of WWP1, along with the down-regulation of Δ Np63 α , in HEK293(Δ Np63 α), MDA-MB-231 and MCF-10A cells (Fig 2A–2C). To study whether this up-regulation of WWP1 occurs at mRNA level, like it was reported previously [20], we measured the mRNA level of WWP1 by means of qRT-PCR. The results revealed that either Doxo or CDDP significantly increases mRNA level of WWP1 (Fig 2D). These results suggest that DNA damage may stimulate transcription of WWP1.

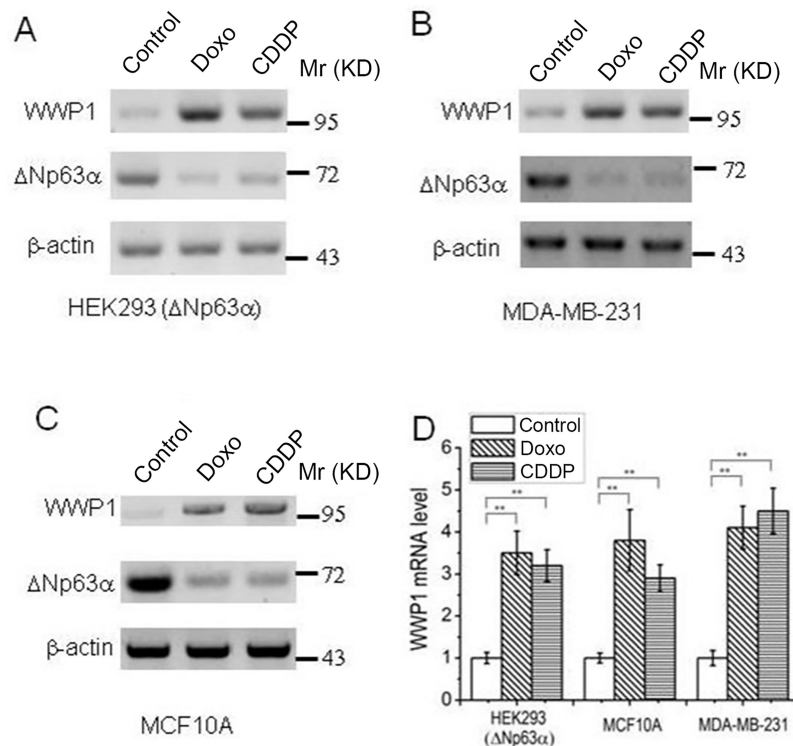


Fig 2. Transcription of WWP1 is induced by DNA damage. Cells were treated with 1 μ M Doxo or 20 μ M CDDP for 48 hours, then subject to IB analysis (A–C) or quantitative reverse transcription-polymerase chain reaction (D). mRNA levels of WWP1 were normalized to those of GAPDH, and presented as means \pm standard deviations (n = 3). P-values were calculated using the Student's *t* test. **, *P* < 0.01.

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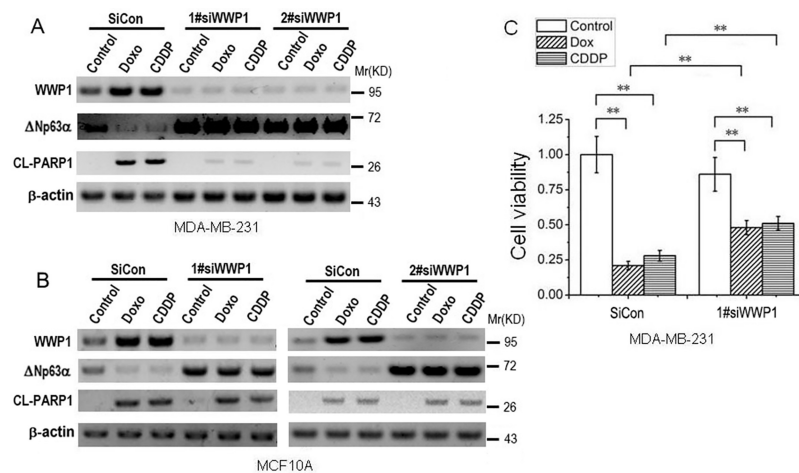


Fig 3. Knock-down of WWP1 abrogates Δ Np63 α destabilization and partially rescues cell apoptosis induced by DNA damage. 6 hours post transfection with siRNAs specific to WWP1 or scrambled control, cells were treated with 1 μ M Doxo or 20 μ M CDDP for 48 hours, then subject to IB analysis (A and B) or MTT assay (C). Cell viability was presented as means \pm standard deviations (n = 3). P-values were calculated using the Student's *t* test. **, *P* < 0.01.

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3. Knock-down of WWP1 abrogates Δ Np63 α destabilization and partially rescues cell apoptosis induced by DNA damage

To investigate if WWP1 is involved in DNA damage-induced down-regulation of Δ Np63 α we knocked down WWP1 with small interfering RNAs (siRNAs). The results showed that compared with its scrambled control (siCon), siRNA-mediated knock-down of WWP1 (siWWP1) significantly up-regulates Δ Np63 α protein level and abrogates DNA damage-induced down-regulation of Δ Np63 α ; concomitantly, DNA damage-induced cleavage of PARP1, which is a hall marker for cell apoptosis, is partially rescued in MDA-MB-231 cells (Fig 3A), while subtle rescue of PARP1 cleavage is observed in MCF10A cells (Fig 3B), which may be due to existence of wild-type and functional p53 in the latter cell type. The results of MTT assay confirmed the rescue effect of siWWP1 on DNA damage-induced cell death in MDA-MB-231 cells (Fig 3C). These results suggest that DNA damage-induced up-regulation of WWP1 may account for Δ Np63 α destabilization in some cell types.

4. p53 or miR-452 may be involved in DNA damage induced-upregulation of WWP1 transcription

It has been reported that DNA damage induces transcription of WWP1 via up-regulation of p53 [20]. In our system, we also found that p53 is up-regulated in MCF10A cells after treatment with DNA damage agents (data not shown). To investigate whether p53 is involved in increase of WWP1 in our system, we employed a siRNA-mediated knock-down of p53. The results reveals that in MCF10A cells, knock-down of p53 induces down-regulation of WWP1 as well as up-regulation of Δ Np63 α , and abrogates Doxo-induced up-regulation of WWP1 and its effect on Δ Np63 α (Fig 4A). However, in MDA-MB-231 cells, Doxo fails to increase p53 despite the up-regulation of WWP1 along with the down-regulation of Δ Np63 α ; on the other hand, knock-down of p53 fails to abrogate effects of Doxo on WWP1 and Δ Np63 α in MDA-MB-231 cells (Fig 4B). This suggests that DNA damage may stimulate WWP1 in these two cell types in different manners.

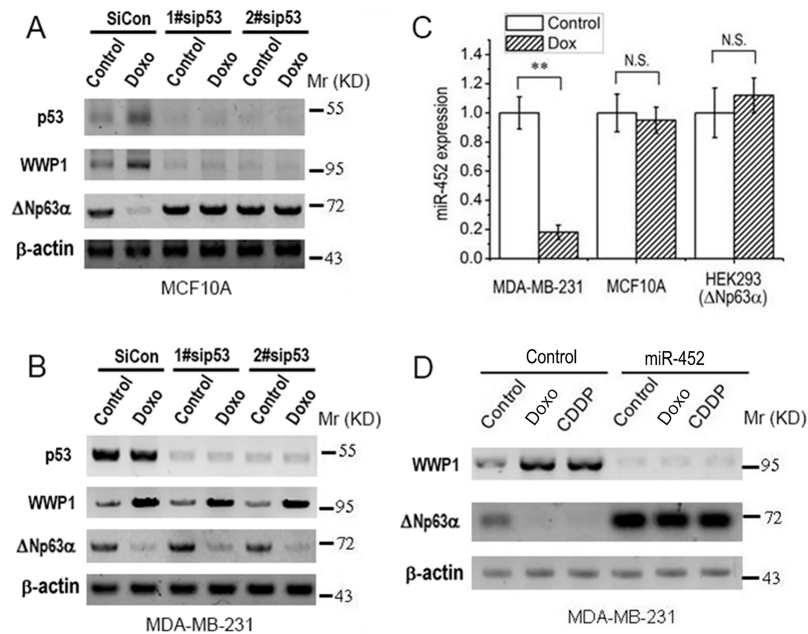


Fig 4. p53 or miR-452 may be involved in DNA damage induced-upregulation of WWP1 transcription. **A** and **B**, 6 hours post transfection with siRNAs specific to p53 or scrambled control, cells were treated with 1 μ M Doxo for 48 hours, then subject to IB analysis. **C**, Cells were treated with 1 μ M Doxo for 48 hours, then subject to qRT-PCR analysis. Expression level of miR-452 was normalized to RNU48 and presented as means \pm standard deviations ($n = 3$). P -values were calculated using the Student's t test. N.S., nonsignificance; **, $P < 0.01$. **D**, 6 hours post transfection with precursor for miR-452 or control, cells were treated with 1 μ M Doxo or 20 μ M CDDP, then subject to IB analysis.

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It was recently reported that WWP1 can be directly inhibited by microRNA-452 (miR-452) [19]. To investigate whether miR-452 is involved in DNA damage-induced up-regulation of WWP1, we measured its mRNA level by means of qRT-PCR and found that after treatment with Doxo, miR-452 is significantly decreased in MDA-MB-231 cells, but not in MCF10A and HEK293(Δ Np63 α) cells (Fig 4C). In another experiment (Fig 4D), we overexpressed miR-452 by transfecting MDA-MB-231 cells with its precursor and found that miR-452 significantly induces down-regulation of WWP1 as well as up-regulation of Δ Np63 α ; miR-452 effectively abrogates the effects of DNA damage agents on either WWP1 or Δ Np63 α in MDA-MB-231 cells. These results suggest that decreased miR-452 may account for up-regulation of WWP1 as well as down-regulation of Δ Np63 α induced by DNA damage in MDA-MB-231 cells.

Discussion

Δ Np63 α is the predominant isoform of p63 gene expressed in epithelial cells [13, 21, 22]. Several E3 ligases, such as Fbw7, Itch1 and WWP1, are reported to target Δ Np63 α to proteasomal degradation [20, 22, 23]. Increasing evidence shows that Δ Np63 α is tightly controlled under differentiation or apoptotic conditions [5, 7, 24–26]. We previously reported that Δ Np63 α inhibits cell apoptosis independently of p53 upon DNA damage: in FaDu and HaCat cells, which carry p53 mutation, Δ Np63 α is significantly down-regulated upon genotoxic stress, resulting in cell apoptosis; this down-regulation of Δ Np63 α induced by DNA damage occurs mainly at mRNA level [17]. In the present study, we used a HEK293 cell line stably expressing transfected Δ Np63 α and two mammary epithelial cell lines, MDA-MB-231 and MCF10A, which express high level of endogenous Δ Np63 α [14, 27]. We found that DNA damage induced

down-regulation of $\Delta Np63\alpha$ also occurs in these cells. Unlike that in FaDu or HaCat cells, DNA damage-induced down-regulation of $\Delta Np63\alpha$ in these three cell lines is attributed to facilitated proteasomal degradation of this protein (Fig 1).

Under different scenarios, E3 ligase WWP1 may execute different roles in regulating $\Delta Np63\alpha$. Although Peschiaroli A *et al.* reported that WWP1 mediates Lys63-linked ubiquitination of $\Delta Np63$ and regulates $\Delta Np63$ -dependent transcription but not its proteasomal degradation in primary human keratinocytes [28], evidence from Li Y *et al.* shows that in some breast cancer cell lines, particularly under DNA damage conditions, WWP1 can mediate proteasomal degradation of $\Delta Np63\alpha$ [20]. According to Li Y's results, DNA damage chemotherapeutic drugs, Doxo and CDDP, induce p53 expression and a concomitant up-regulation of WWP1 mRNA level in cells carrying wild-type p53; absence of wild-type p53 abrogates DNA damage-induced increase in WWP1 mRNA and protein [20]. In line with these observations, we found that either Doxo or CDDP induces significant increase in mRNA and protein levels of WWP1 (Fig 2), whose depletion can abrogate DNA damage-induced down-regulation of $\Delta Np63\alpha$ in our system (Fig 3). In MCF10A cells, which carry wild-type p53 [14], DNA damage stimulates WWP1 expression via up-regulation of p53, of which underlying mechanism remains unknown (Fig 4A). However, in MDA-MB-231 cells, which carry mutant p53 [27], this mutant p53 does not seem to respond to DNA damage; and knock-down of p53 fails to abrogate DNA damage-induced up-regulation of WWP1 in these cells (Fig 4B). On the other hand, in MDA-MB-231 but not MCF10A or HEK293($\Delta Np63\alpha$) cells, DNA damage induces significant down-regulation of microRNA-452 (Fig 4C), which is recently reported as a regulatory factor of WWP1 expression [19]; and overexpression of microRNA-452 depletes WWP1 in MDA-MB-231 cells, resulting in up-regulation of $\Delta Np63\alpha$ as well as abrogation of DNA damage-induced $\Delta Np63\alpha$ down-regulation (Fig 4D). These results indicate that DNA damage induces WWP1 via p53 in some cells carrying wild-type p53 such as MCF10A and HEK293, or likely via miR-452 in cells carrying mutant p53 such as MDA-MB-231.

Taken together, we demonstrated that protein level of $\Delta Np63\alpha$ can be down-regulated in different pathways in response to genotoxic stress. On one hand, DNA damage may negatively regulate $\Delta Np63\alpha$ via inhibiting its transcription [17]. On the other hand, DNA damage can also induce proteasomal degradation of $\Delta Np63\alpha$ via stimulating WWP1 E3 ligase in different manners.

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Author Contributions

Conceptualization: CL.

Formal analysis: JC HS DL.

Investigation: JC HS YC SF.

Writing – original draft: JC HS CL.

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